

**APPLICATION FOR UNITED STATES LETTERS PATENT
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Inventors: Laurence A. Cole
2315 Via Seville Ct, NW
Albuquerque, NM 87104

A Citizen of the United States

Assignee: University of New Mexico
Albuquerque, NM

Title: Hyperglycosylated hCG (Invasive Trophoblast Antigen) In
Differential Diagnosis of Malignant or Invasive Trophoblastic
Disease

Hyperglycosylated hCG (Invasive Trophoblast Antigen) In Differential Diagnosis of Malignant or Invasive Trophoblastic Disease

This application is related to provisional application Serial No. 60/418,128 filed
5 October 10, 2002.

FIELD OF THE INVENTION

The invention relates to methods and reagents for differential diagnosis of
quiescent and invasive gestational trophoblastic diseases. The invention also relates to
10 methods for detecting the presence or absence of germ cell tumors.

BACKGROUND OF THE INVENTION

A gestational trophoblastic disease is characterized by abnormal cellular growth
of the tissues formed as a result of conception, leading to the development of tumors
15 within the uterus. There are three types of gestational trophoblastic tumors: hydatidiform
mole/molar pregnancy, choriocarcinoma, and placenta-site trophoblastic tumor (PSTT).
Typically, a gestational trophoblastic disease is diagnosed by ultrasound or a
Computerized Axial Tomography (CT) or Magnetic Resonance Imaging (MRI) imaging
methods. The key test, however, is the hCG blood test.

20 hCG (human chorionic gonadotropin) is a hormone that increases during
pregnancy and is commonly used to confirm pregnancy and as a marker for gestational
trophoblastic disease and germ cell tumors. If a woman is not pregnant but has an
increased level of hCG it may indicate the presence of a gestational trophoblastic tumor.
hCG levels are often rechecked multiple times if a trophoblastic tumor is suspected, since
25 they are a direct measure of the amount of tumor tissue present.

Typically, treatment for gestational trophoblastic disease includes chemotherapy,
surgery and combination chemotherapy, or a combination thereof. The treatment or

combination of treatments that is recommended will depend upon the severity of the disease. A gestational trophoblastic disease can be invasive, which will require a more rigorous treatment regimen, or non-invasive, which will not require any of the treatments mentioned above. In fact, when non-invasive or slow growing, none of the above
5 mentioned treatments would be effective since they act on fast growing tumors. However, there is currently no accurate method for distinguishing between invasive and non-invasive gestational trophoblastic disease. Thus, there is a need in the art for a method of diagnosing non-invasive gestational trophoblastic disease in order to spare a patient from undergoing unnecessary treatments used to treat invasive gestational
10 trophoblastic disease.

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods for detecting the presence or absence of invasive trophoblast cells comprising the steps of: (a) obtaining a biological
15 sample from a patient; (b) measuring an amount of hCG in the biological sample; (c) measuring an amount of ITA in the biological sample; and (d) determining the percentage of hCG that is ITA, wherein invasive trophoblast cells are detected if the percentage of hCG that is ITA is 30% or greater.

In one embodiment, the patient is a patient in which persistently low hCG titers
20 have been previously detected, and wherein quiescent gestational trophoblastic disease is diagnosed if the percentage of ITA relative to hCG determined in step (d) is less than 30%.

In a further aspect, the invention provides methods for detecting the presence or absence of invasive trophoblast cells in a biological sample comprising the steps of: (a)
25 obtaining a biological sample from a patient; and (b) measuring an amount of ITA in the biological sample; wherein invasive trophoblast cells are detected if the amount of ITA in the biological sample is 2 IU/L or greater.

In another aspect, the invention provides methods for monitoring progression of quiescent gestational trophoblastic disease comprising the steps of: (a) obtaining a

biological sample from a patient diagnosed as having quiescent gestational trophoblastic disease; (b) measuring an amount of hCG in the biological sample; (c) repeating steps (a) and (b) with a biological sample obtained at one or more subsequent time points; (d) measuring an amount of ITA in a biological sample obtained in step (c) where the
5 amount of hCG in the biological sample from step (c) is higher than the amount of hCG in step (b); and (e) determining a percentage of hCG that is ITA in the biological sample from step (d).

In yet another aspect, the invention provides methods of detecting the presence or absence of a germ cell tumor in a biological sample comprising the steps of: (a) obtaining
10 a biological sample from a patient; (b) measuring an amount of hCG in the biological sample; (c) measuring an amount of ITA in the biological sample; and (d) determining the percentage of hCG that is ITA, wherein a germ cell tumor is detected if the percentage is 30% or greater.

In a further aspect, the invention provides methods of detecting the presence or
15 absence of a germ cell tumor in a biological sample comprising the steps of: (a) obtaining a biological sample from a patient; and (b) measuring an amount of ITA in the biological sample; wherein a germ cell tumor is detected if the amount of ITA in the biological sample is 2 IU/L or greater.

In another aspect, the invention provides methods of monitoring the progression
20 of a germ cell tumor comprising the steps of: (a) obtaining a biological sample from a patient diagnosed as having a germ cell tumor; (b) measuring an amount of hCG in the biological sample; (c) repeating steps (a) and (b) with a biological sample obtained at subsequent time points; (d) measuring an amount of ITA in a biological sample from step (c) if the amount of hCG in a biological sample from step (c) is higher than the amount of
25 hCG in step (b); and (e) determining the percentage of hCG that is ITA in the biological sample from step (d).

In one embodiment, a germ cell tumor detected or monitored by a method of the invention can be an ovarian germ cell tumor or a testicular germ cell tumor.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chart on use of invasive trophoblastic antigen (ITA) as a tumor marker in selected patient populations. Across the X axis: (1) represents the percentages of ITA in 38 patients with persistent low levels of hCG as described in Table 1; (2) represents the percentages of ITA in 4 patients re-referred to the USA hCG Reference Service, all of whom initially presented with persistent low levels of hCG and were either shown or clinically suspected of having malignant gestational trophoblastic disease when sudden rapidly rising hCG levels were detected; and (3) represents 15 other cases with proven gestational trophoblastic disease (choriocarcinoma or invasive placental site trophoblastic tumor). The value 100% indicates that the ITA assay was >99% of that in the total hCG assay (which measures all forms of hCG including ITA). ITA units are equal on a mass basis to hCG units (IU/L).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, "hCG" is an abbreviation for human chorionic gonadotropin, a glycoprotein hormone secreted in relatively large quantities by the trophoblast cells of the placenta. The term "intact" or "regular" hCG is used herein to refer to hCG that is composed of two dissimilar subunits, α (92 amino acids and two N-linked oligosaccharides) and β (145 amino acids and two N-linked and four O-linked oligosaccharides), that are joined noncovalently. Both subunits are extensively glycosylated leading to a heterogeneous population of molecular forms. The pattern of glycosylation varies somewhat between sources due to differences in the available processing enzymes. hCG can be detected in the serum and urine of pregnant women and in those with gestational trophoblastic disease.

Free α - and free β -subunits are also detected in serum and urine samples. Both subunits undergo extensive glycosylation, creating a mixture of various forms of hCG and free hCG subunits in a sample. Furthermore, free β subunit and β subunits of intact hCG are frequently cleaved at position 47-48 to produce nicked hCG and nicked β subunit, which are biologically inactive. Further degradation of nicked free β subunit yields a β -core fragment, which forms a large proportion of β subunit-related molecules in urine. For further review of hCG and various forms of hCG, see, for example, Norman *et al.*, 1990, *Ann. Clin. Biochem.* 27:183-194; Cole, 1997, *Clin. Chem.* 43:2233-2243; and Sturgeon *et al.*, 1998, *Ann. Clin. Biochem.* 35:460-491.

As used herein, "ITA" is an abbreviation for invasive trophoblast antigen, also known as hyperglycosylated hCG. ITA is, therefore, a variant of regular hCG, comprising additional side chains of sugars on the N-linked and O-linked sugar chains compared with regular hCG. These additional sugar side chains comprise N-acetylglucosamine, galactose and sialic acid and make the molecule considerably larger in size. ITA is best detected with an immunoassay using the specific monoclonal antibody B152, which only detects ITA. Other methods for detecting ITA include carbohydrate analysis, isoelectric focusing, and the use of lectins, which can distinguish ITA from other forms of hCG based on the carbohydrate units attached to ITA. As discussed herein, ITA is produced by invasive cytotrophoblast cells and malignant germ cells. Typically, measuring ITA levels in a patient who is not pregnant and/or is free of a disease that has cells producing ITA, will yield a background level of ITA of less than 2 IU/L. Thus, as described herein, where a patient is not pregnant, an ITA level of 2 IU/L or greater is indicative of a cancer. In a particular embodiment, where a patient is not pregnant, a level of ITA that is 2 IU/L or greater in a biological sample can indicate the presence of invasive trophoblast cells and/or a germ cell tumor, wherein a level of ITA that is lower than 2 IU/L can indicate the absence of invasive trophoblast cells and/or a germ cell tumor.

The term "hCG" is used herein to refer to any subunit of hCG, fragment of hCG, intact hCG, an isoform of hCG, a modified hCG molecule, total hCG, or any combination thereof. A subunit of hCG can be, for example, α or β subunits. A fragment of hCG can

be, for example, β -core fragment or hCG missing the β -subunit C-terminal peptide. A modified hCG molecule can be, for example, nicked free β -subunit or nicked intact hCG. An isoform of hCG can be, for example, ITA.

As used herein, "total hCG" comprises regular hCG and all forms of hCG, including free α and β subunits of hCG and β core fragment of hCG, as well as hCG isoforms such as ITA.

The term "quiescent gestational trophoblastic disease" is used herein to describe a dormant syndrome characterized by non-invasive or quiescent trophoblastic tissue, failed response to chemotherapy and surgery, and the retention of some malignant potential.

The term "gestational trophoblastic disease," as used herein, includes hydatidiform mole/molar pregnancy, choriocarcinoma, and placenta-site trophoblastic tumor.

The term "germ cell" tumor, as used herein, refers to a tumor arising from ovarian germ cells or testicular germ cells. Germ cell tumors that produce ITA include, for example, dysgerminoma and other ovarian germ cell tumors, and testicular choriocarcinoma and seminoma. In one embodiment of the invention, ITA is a useful marker in detecting invasive disease in all these germ cell tumors or malignancies.

The term "biological sample", as used herein, includes, but is not limited to, a quantity of a tissue or body fluid sufficient to measure hCG and/or ITA levels, that is obtained from a mammal. In one embodiment, the mammal is a human. Preferably, a biological sample is urine, saliva, cells, plasma, or serum. Most preferably, a biological sample is urine.

As used herein, the term "patient" includes mammalian subjects. Preferably, a patient is a human. In one embodiment, the patient is a male or female in which persistently low hCG titers have been previously detected, a female diagnosed as having quiescent gestational trophoblastic disease, a female at risk of having or suspected of having a gestational trophoblastic disease, or a male or female at risk of having or suspected of having or in need of screening for a germ cell tumor.

As used herein, a "low level" of hCG is detected where the concentration of hCG is 2 to 200 IU/L. A "persistently" low level of hCG is detected where a low level of hCG is detected in biological samples obtained from a single patient at subsequent time intervals, for example, at weekly, monthly, or bi-monthly time intervals.

5 As used herein, the term "detecting" refers to identifying a patient with invasive trophoblast cells or a germ cell tumor and/or a patient who is at high risk for having an invasive gestational trophoblastic disease or a germ cell tumor.

10 In one aspect, the invention provides a method of detecting the presence or absence of invasive trophoblast cells comprising the steps of: (a) obtaining a biological sample from a patient; (b) measuring an amount of hCG in the biological sample; (c) measuring an amount of ITA in the biological sample; and (d) determining a percentage of hCG that is ITA, wherein invasive trophoblast cells are detected if the percentage of hCG that is ITA is at least 30%. In one embodiment, the percentage of hCG that is ITA can be at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%,
15 or 100%. In a preferred embodiment, invasive trophoblast cells are detected if the percentage of hCG that is ITA is at least 30% or greater. In one embodiment, hCG can be regular hCG, a subunit of hCG, an isoform of hCG, a fragment of hCG, a combination thereof, or total hCG.

20 Methods for measuring an amount of hCG in a sample are known in the art, and include, for example, using commercially available hCG tests. Such tests have been described, for example, in Cole, 1998, *Gynecol. Oncol.* 71:325-329; Rotmensch *et al.*, 2000, *Lancet* 355:712-715; Cole *et al.*, 2001, *Clin. Chem.* 47:308-315; Cole *et al.*, 2001, *Clin. Lab. Intl.* 25:9-14; Cole *et al.*, 2002, *J. Reprod. Med.* 47:433-444; Butler *et al.*, 2001, *Clin. Chem.* 47:1332-1333; Butler *et al.*, 2002, *Obstet. Gynecol.* 99:515-516; Cole
25 *et al.*, 1999, *Clin. Chem.* 45:2109-2119; all of which are hereby incorporated by reference. The DPC Immulite hCG test (DPC Biermann, Germany) is particularly useful for detecting total hCG.

Methods for measuring an amount of ITA include, for example, methods described in U.S. Patent No. 6,429,018 (which is hereby incorporated by reference), ITA

kits that are available, for example, from Quest Diagnostics (Teterboro, NJ) and Nichols Institute Diagnostics (San Clemente, CA), and methods described herein.

Any assay that functions to qualitatively or quantitatively determine concentrations of hCG in a biological sample and/or detects ITA concentrations can be employed in the practice of the invention. A direct assay, such as an immunoassay using antibodies that recognize hCG, free hCG subunits, the hCH β -core fragment, and/or specific hCG isoforms, is preferred. Other exemplary assays can involve lectins that assay for carbohydrate moieties, chromatography, chemical or electrophoresis or isoelectric focusing tests that detect glycosylation variants of hCG, and/or antibodies to hyperglycosylated or carbohydrate-variant hCG. Such assays are described in the art.

Immunoassays that can be used to detect hCG or ITA include, but are not limited to, assays employing specific antibodies to hCG or ITA, and assays employing nonspecifically defined antibodies obtained by blind injections of a hCG or ITA into test animals using standard methods. Antibodies to hCG and ITA can be generated by standard means as described, for example, in "Antibodies: A Laboratory Manual" by Harlow and Lane (Cold Spring Harbor Press, 1988), which is hereby incorporated by reference. Any type of fusion phage, monoclonal, or polyclonal antibodies can be used in immunoassays of the invention, so long as the antibodies can be used in a reproducible fashion as markers for hCG or ITA.

In one embodiment, an amount of hCG or ITA can be measured using a capture antibody followed by a labeled secondary antibody using a strategy as described, for example, in U.S. Patent No. 6,429,018 (which is hereby incorporated by reference). U.S. Patent No. 6,429,018 teaches the B152 antibody that recognizes ITA but does not detect normal hCG. A labeled secondary antibody useful in a method of the invention can be, for example, an anti-hCG antibody, β -core fragment, α -subunit, and/or β -subunit, providing an assay with polypeptide specificity. The label on the secondary antibody can comprise any chemical, radioactive, lanthanide, colored dye, or genetic tag used in enzyme-linked immunosorbent assays (ELISAs), Western blots, and other sensitive and specific immunoassays and immunoradiometric assays using known methodology. These include conjugating the antibody with horseradish peroxidase or alkaline phosphatase that

are easily measurable, typically using colorimetric, fluorometric or luminescent substrates. Genetic labels include firefly luciferase, employed because luciferase produces a bioluminescent molecule when incubated with its substrate, luciferin.

5 In other embodiments, hCG peptide-specific antibody can be used as a capture antibody, and an antibody specific to ITA can be used as the secondary labeled antibody in an immunoassay such as those described above. Competitive immunoassays can also be used to determine the amount of ITA present among other forms of hCG, including regular and total hCG. For example, an antibody that binds non-specifically to both intact hCG and ITA can be used to immunoprecipitate both forms of hCG present in a
10 sample, followed by a second immunoprecipitation step in which ITA-specific antibodies are used to separate ITA from regular hCG, free hCG subunits, and hCG β core fragment. Isolated ITA protein can then be quantified using standard methods, such as a Bradford protein assay. Alternate embodiments using concanavalin A or other carbohydrate-specific lectin can be used in place of the capture antibody or labeled antibody.
15 Alternatively, prior to an immunoassay, a lectin or chromatographic method can be used to extract ITA from a biological sample. Lectins bind specific carbohydrate structures. Thus, they can be used to differentiate ITA and other forms of hCG, including regular hCG and various hCG subunits or hCG fragments. ITA is also slightly larger than regular hCG and can be separated from regular hCG or other hCG forms by
20 chromatography or electrophoresis.

Carbohydrate analyses include qualitative observations of differences in physical properties between regular hCG and ITA or subunits and fragments thereof, carbohydrate identification using plant lectins specific to the variant carbohydrate portion of ITA obtained by standard lectin screening methods, or any other fingerprinting technique
25 including qualitative or quantitative carbohydrate composition analyses. See, for example, U.S. Patent No. 6,429,018, which is incorporated by reference.

In certain embodiments, hCG can be purified from biological samples prior to determining the percentage of hCG that is ITA from the sample. Any method for purifying hCG can be used. For example, antibodies specific for hCG can be used to
30 isolate hCG from a biological sample. The amount of ITA in a purified hCG protein

fraction can be determined using a method as described above, for example, using an immunoassay with antibodies specific for ITA. ITA specific antibodies are described, for example, in U.S. Patent No. 6,429,018, which is hereby incorporated by reference. Purified hCG and hCG isoforms from control or test samples can be stored under
5 appropriate conditions, such as those described, for example, in Cole *et al.*, 1999, *Clinical Chem.* 45:2109-2119, which is hereby incorporated by reference.

One preferred method for determining the amount of ITA in a biological sample is described in U.S. Patent No. 6,429,018, which teaches a two-step sandwich immunoassay using B152 as capture antibody, and peroxidase labeled monoclonal
10 antibody to hCG β -core, batch 4001 (Genzyme, San Carlos, California) as tracer using the immunoassay methods previously described (Elliott, *et al.*, 1997, *Endocrine* 7:15-32; and Cole *et al.*, 1993, *J. Clin. Endocrinol. Metab.* 76:704-710).

In another aspect, the invention provides a method of diagnosing quiescent gestational trophoblastic disease or invasive gestational trophoblastic disease in a patient
15 comprising the steps of: (a) obtaining a biological sample from a patient, wherein the patient has persistently low hCG titers; (b) measuring an amount of hCG in the biological sample; (c) measuring an amount of ITA in the biological sample; and (d) determining the percentage of hCG that is ITA, wherein quiescent gestational trophoblastic disease is diagnosed if the percentage determined in step (d) is less than 30%, and invasive
20 gestational trophoblastic disease is diagnosed if the percentage determined in step (d) is greater than 30%. In one embodiment, hCG can be regular hCG, a subunit of hCG, an isoform of hCG, a fragment of hCG, a combination thereof, or total hCG.

Alternatively, the amount of ITA from step (c) can be compared to a predetermined value, wherein the patient is diagnosed as having invasive gestational
25 trophoblastic disease if the amount of ITA is equal to or greater than the predetermined value, and wherein the patient is diagnosed as having quiescent gestational trophoblastic disease if the amount of ITA is less than the predetermined value. In a particular embodiment, the predetermined value is 2 IU/L, as discussed above. Certain embodiments of this aspect are as defined above, including the definition of patient and

persistently low levels of hCG, biological sample, quiescent trophoblastic disease, ITA, and hCG.

As used herein, a “predetermined value” is a standardized value based on a control. For example, a predetermined value can be based on an amount of ITA that is present in a biological sample obtained from a woman previously diagnosed with quiescent gestational trophoblastic disease, *i.e.* a woman having persistently low hCG levels without a sharp rise in hCG levels. A “persistently low hCG levels” are defined above. A “sharp rise” in hCG levels occurs when an hCG level observed in a biological sample is at least 2 fold greater than an hCG level observed in a biological sample obtained from the same patient at an earlier time point. For example, a sharp rise is observed where hCG levels increase from 5 IU/ml in one biological sample to 10 IU/ml in a subsequently obtained biological sample, wherein the two biological samples were obtained from the same patient.

Alternatively, the “predetermined value” can refer to a value that is based on an amount of ITA in a biological sample obtained from a woman who has confirmed invasive gestational trophoblastic disease, *i.e.* a woman who has been pathologically diagnosed as having choriocarcinoma. In this embodiment of the invention, the patient is diagnosed as having invasive gestational trophoblastic disease if the amount of ITA is equal to or greater than the predetermined value.

In yet another aspect, the invention provides methods for monitoring the progression of quiescent gestational trophoblastic disease comprising the steps of: (a) obtaining a biological sample from a patient; (b) measuring an amount of hCG in the biological sample; (c) repeating steps (a) and (b) with a biological sample obtained at subsequent time points; (d) measuring an amount of ITA in the biological sample if the amount of hCG in the biological sample from step (c) is higher than the amount of hCG in step (b); and (e) determining the percentage of hCG that is ITA in the biological sample. In one embodiment, the patient can be a patient who was previously diagnosed as having quiescent gestational trophoblastic disease, as diagnosed, for example, using a method of the invention. Alternatively, the patient can be a patient who was previously treated for an invasive gestational trophoblastic disease and whose hCG levels

subsequently decreased relative to hCG levels measured prior to treatment for invasive gestational trophoblastic disease. Certain embodiments of this aspect are as defined above, including the definition of patient and persistently low levels of hCG, biological sample, quiescent trophoblastic disease, ITA, and hCG.

- 5 In another embodiment, steps (a) and (b) can be repeated, for example, daily, weekly, monthly, bi-monthly, every other month, every three months, every four months, every six months, or yearly. In one embodiment, where hCG levels observed over time rise then plateau, quiescent disease is indicated. For example, quiescent disease is indicated where hCG levels rise to 20 IU/ml, then plateau at 22, 18, 25, and 24 IU/ml.
- 10 Where hCG levels rise consistently or sharply, invasive disease is indicated. For example, invasive disease is indicated where hCG levels rise to 20 IU/ml, then continue to rise to 100 IU/ml then 600 IU/ml. In one embodiment, invasive disease is confirmed in a patient having consistently or sharply rising hCG levels by determining the percentage of ITA relative to the total amount of hCG present in a biological sample from
- 15 the patient. Where the amount of ITA is at least 30% or higher, invasive disease is diagnosed. In one embodiment, hCG can be regular hCG, a subunit of hCG, an isoform of hCG, a fragment of hCG, a combination thereof, or total hCG.

 The methods of the invention can be used to diagnose and/or monitor progression of quiescent gestational trophoblastic disease or invasive gestational trophoblastic

20 disease. One recommended management of quiescent disease is close surveillance without therapy until invasive disease is detected. Specifically, the invention provides the following guidelines for the management and monitoring of quiescent gestational trophoblastic disease.

1. A patient having persistent low levels of serum hCG and/or an ITA amount
- 25 that is less than 30% of the patient's hCG is given the diagnosis of quiescent gestational trophoblastic disease instead of invasive or malignant gestational trophoblastic disease.
2. Clinicians should refrain from treating quiescent gestational trophoblastic disease cases with chemotherapy, hysterectomy or other surgery.

3. Since quiescent gestational trophoblastic disease cases can develop into an invasive gestational trophoblastic disease, a patient with quiescent gestational trophoblastic disease should be monitored for an abrupt rise in hCG titers, ITA titers, and/or ITA to hCG ratios, which could occur months or years after the
5 detection of persistent low-level hCG titers.

In yet another aspect, the invention provides methods of detecting the presence or absence of a germ cell tumor in a biological sample comprising the steps of: (a) obtaining a biological sample from a patient; (b) measuring an amount of hCG in the biological sample; (c) measuring an amount of ITA in the biological sample; and (d) determining
10 the percentage of hCG that is ITA, wherein a germ cell tumor is detected if the percentage is 30% or greater. In one embodiment, hCG can be regular hCG, a subunit of hCG, an isoform of hCG, a fragment of hCG, a combination thereof, or total hCG.

In a further aspect, the invention provides methods of detecting the presence or absence of a germ cell tumor in a biological sample comprising the steps of: (a) obtaining
15 a biological sample from a patient; and (b) measuring an amount of ITA in the biological sample; wherein a germ cell tumor is detected if the amount of ITA in the biological sample is 2 IU/L or greater.

In another aspect, the invention provides methods of monitoring the progression of a germ cell tumor comprising the steps of: (a) obtaining a biological sample from a
20 patient diagnosed as having a germ cell tumor; (b) measuring an amount of hCG in the biological sample; (c) repeating steps (a) and (b) with a biological sample obtained at subsequent time points; (d) measuring an amount of ITA in a biological sample from step (c) if the amount of hCG in a biological sample from step (c) is higher than the amount of hCG in step (b); and (e) determining the percentage of hCG that is ITA in the biological
25 sample from step (d). In one embodiment, hCG can be regular hCG, a subunit of hCG, an isoform of hCG, a fragment of hCG, a combination thereof, or total hCG.

EXAMPLES

The following example, including the experiments conducted and results achieved is provided for illustrative purposes only and are not to be construed as limiting the invention.

5

Example 1

ITA tests differentiate quiescent gestational trophoblast disease from invasive gestational trophoblast disease

Samples received in a reference laboratory were initially tested using the automated DPC Immulite total hCG test, the DPC Immulite free hCG β test, Abbott AxSym total hCG test, and other commercial hCG tests as needed. Serum and urine samples were also analyzed using specialized assays. These included the microtiter plate intact hCG only enzyme immunometric assay, the ITA enzyme immunometric assay, the nicked hCG only enzyme immunometric assay, the free hCG β -subunit only enzyme immunometric assay, and the hCG β -subunit core fragment only enzyme immunometric assay. These specialized tests have been described in detail in previous publications (1-8). Tests were run with and without the Scantibodies, Inc. (San Diego, CA), heterophilic antibody blocking agent to detect substances that falsely elevate hCG titers. Different tests were run as needed, including Sigma Diagnostics (St. Louis, MO) urine creatinine test and the automated DPC Immulite LH test (1-8). Clinical reports were prepared from the abbreviated patient history, from the patient's hCG test record, generated data with major commercial automated and "in house" specialized assays, and published data (1-7).

Table 1 outlines the clinical histories provided for the 63 patients with persistent low-level hCG determinations. The table shows the antecedent gestation, the period of persistent low serum hCG results, the limits of serum hCG results as determined by the referring physician's laboratory and the reference laboratory, and therapy used for treating the low hCG results, as indicated in reports to the reference laboratory.

Table 1. Histories of 63 Cases with Persistent Low Levels of hCG				
Case	Antecedent Gestation	Period of low serum hCG results	Limits of serum hCG Results	Therapy used after identification of persistent low hCG results
1	hydatidiform mole	<½ year	6 - 37 IU/L	HYS, BSO, EMA-CO, Taxol, Carbo
2	pregnancy	2½ year	13 - 60 IU/L	Mtx, Act-D, HYS, BSO, EMA-CO
3	hydatidiform mole	>½ year	5 - 10 IU/L	Act-D, Mtx, HYS, EMA-CO
4	pregnancy	3 year	18 - 150 IU/L	HYS, BSO, Act-D, EMA-CO
5	pregnancy	3½ year	11 - 19 IU/L	Mtx, Act-D, HYS, BSO, Act-D
6	hydatidiform mole	1½ year	<2 - 39 IU/L	Act-D, EMA-CO, EIP, EIC
7	pregnancy	4½ year	37 - 198 IU/L (863 IU/L)*	MtxLv, HYS, BSO, Mtx
8	pregnancy	6 year	44 - 198 IU/L	HYS, Mtx, EMA-CO
9	hydatidiform mole	unavailable	100 - 400 IU/L	HYS, BSO, Act-D
10	pregnancy	2 year	<2 - 90 IU/L	Mtx, Act-D, EMA-CO
11	pregnancy	3 year	<1 - 100 IU/L	Mtx, HYS
12	hydatidiform mole	1 year	6 - 54 IU/L	HYS
13	pregnancy	1 year	60 - 130 IU/L	HYS
14	hydatidiform mole	3 year	<2 - 59 IU/L (6000 IU/L)†	BSO, Mtx
15	hydatidiform mole	< 1 year	2 - 37 IU/L	Mtx, Mtx, Act-D
16	hydatidiform mole	<½ year	<5 - 12 IU/L	Act-D, Mtx
17	hydatidiform mole	6 year	7 - 35 IU/L	Mtx, Act-D
18	pregnancy	2 year	17 - 148 IU/L (355 IU/L)‡	Mtx, Act-D
19	hydatidiform mole	1½ year	<2 - 41 IU/L	Mtx, Act-D
20	pregnancy	1 year	54 - 500 IU/L	Mtx, Act-D
21	hydatidiform mole	<½ year	7 - 48 IU/L	Mtx, Act-D
22	pregnancy	<½ year	7 - 35 IU/L	Mtx, Mtx
23	hydatidiform mole	1 year	36 - 66 IU/L	Mtx, Act-D

24	pregnancy	<½ year	12 - 42 IU/L	Mtx, Mtx
25	hydatidiform mole	<½ year	7 - 34 IU/L	Mtx, Act-D
26	hydatidiform mole	1½ year	8 - 35 IU/L	5FU
27	hydatidiform mole	4½ year	2 - 7 IU/L	Act-D
28	hydatidiform mole	<½ year	19 - 32 IU/L	Mtx
29	pregnancy	<½ year	165 - 224 IU/L	Mtx
30	pregnancy	<½ year	34 - 56 IU/L	Mtx
31	pregnancy	½ year	100 - 174 IU/L	Mtx
32	hydatidiform mole	1 year	6 - 13 IU/L	Mtx
33	pregnancy	<½ year	10 - 12 IU/L	Mtx
34	hydatidiform mole	1 year	25 - 37 IU/L (489 IU/L)§	Mtx
35	pregnancy	2 year	50 - 200 IU/L	Mtx
36	hydatidiform mole	1 year	15 - 33 IU/L	Mtx
37	pregnancy	1 year	2 - 485 IU/L	chemotherapy, details unavailable
38	hydatidiform mole	1 year	<2 - 23 IU/L	chemotherapy, details unavailable
39	pregnancy	2 year	7 - 22 IU/L	chemotherapy, details unavailable
40	hydatidiform mole	1 year	8 - 23 IU/L	chemotherapy, details unavailable
41	pregnancy	<½ year	157 - 206 IU/L	no new or further therapy started
42	hydatidiform mole	2½ year	32 - 100 IU/L	no new or further therapy started
43	pregnancy	<½ year	5 - 8 IU/L	no new or further therapy started
44	hydatidiform mole	½ year	6 - 12 IU/L	no new or further therapy started
45	pregnancy	1 year	8 - 21 IU/L	no new or further therapy started
46	hydatidiform mole	<½ year	49 - 64 IU/L	no new or further therapy started
47	pregnancy	3 year	4 - 7 IU/L	no new or further therapy started
48	hydatidiform mole	1 year	11 - 34 IU/L	no new or further therapy started

49	pregnancy	<½ year	58 - 146 IU/L	no new or further therapy started
50	hydatidiform mole	½ year	14 - 21 IU/L	no new or further therapy started
51	pregnancy	<1 year	5 - 10 IU/L	no new or further therapy started
52	hydatidiform mole	1 year	12 - 44 IU/L	no new or further therapy started
53	pregnancy	2 year	10 - 40 IU/L	no new or further therapy started
54	pregnancy	<½ year	223 - 496 IU/L	no new or further therapy started
55	pregnancy	<½ year	6 - 9 IU/L	no new or further therapy started
56	pregnancy	<½ year	6 - 7 IU/L	no new or further therapy started
57	pregnancy	5½ year	23 - 52 IU/L	no new or further therapy started
58	pregnancy	<½ year	8 - 11 IU/L	no new or further therapy started
59	pregnancy	<½ year	3 - 28 IU/L	no new or further therapy started
60	GTN	<½ year	<2 - 57	no new or further therapy started
61	pregnancy	<½ year	10 - 148	no new or further therapy started
62	pregnancy	<½ year	49 - 69 IU/L	No new or further therapy started
63	pregnancy	<½ year	13 - 91 IU/L	No new or further therapy started

HYS, hysterectomy; *BSO*, bilateral salpingoopharectomy; *Carbo*, carboplatin; *Mtx*, methotrexate; *Act-D*, actinomycin D; *EMA-CO*, combination etoposide, methotrexate, actinomycin D alternating with cyclophosphamide, and vincristine chemotherapy; *Taxol*, paclitaxel; *EIP*, combination etoposide, ifosfamide, and cisplatin; *EIC*, combination etoposide, ifosfamide, and carboplatin; *MtxLv*, methotrexate with leucovorin rescue; *5FU*, 5-fluorouracil; *Mtx, Mtx*, 2 separate treatments with methotrexate; *GTN*, gestational trophoblastic neoplasm.

*After 4½ years of persistent low levels (*ITA* not detected), *hCG* suddenly rose sharply, and patient was re-referred; *hCG* was then 863 IU/L, 86% of *hCG* immunoreactivity due to *ITA* (Figure 1). Invasive gestational trophoblastic neoplasm indicated.

† After 3 years of persistent low levels of *hCG* (*ITA* not detected) values rose sharply, and patient was re-referred; *hCG* result was then 6000 IU/L. At this time 81% of *hCG* immunoreactivity due to *ITA* (Figure 1). Invasive gestational trophoblastic neoplasm indicated.

[†]After 2 years persistent low levels of hCG results, values sharply increased and patient was re-referred; hCG result was then 355 IU/L. At that time 100% of hCG immunoreactivity due to ITA (Figure 1); patient was found to have invasive placental trophoblastic tumor.

[§]After 1 year of persistent low levels of hCG (ITA not detected) values rose sharply, and patient was re-referred; hCG result was then 489 IU/L. At this time 91% of hCG immunoreactivity due to ITA (Figure 1). Invasive gestational trophoblastic neoplasm indicated.

In all 63 cases, there was no clinical or radiographic evidence of pregnancy or gestational trophoblastic disease or gestational trophoblastic neoplasm. As illustrated, patients with measurable low-level persistent hCG titers were followed for variable time periods (less than 6 months to over 6 years). Intact hCG, free β -subunit, and β -subunit core fragment were measured in serum and urine in all 63 cases. In all cases intact hCG accounted for >90% of combined immunoreactivity. This combination is similar to the normal pregnancy balance of hCG and degradation products. Of note, in 38 (60%) cases, the hCG titers never exceeded 50 IU/L. The clinically recognized pregnancy event before this presentation, referred to in this report as the antecedent pregnancy, was a hydatidiform mole in 27 cases (43%); 35 (56%) had a term, ectopic, or spontaneously aborted pregnancy; one case had a past history of gestational trophoblastic neoplasm.

In all 63 cases, the broad presumptive diagnosis of gestational trophoblastic disease was made, and 40 (64%) underwent single-agent or combination chemotherapy, hysterectomy, or both. Twelve patients underwent hysterectomy and/or bilateral salpingo-oophorectomy, including 10 who also received chemotherapy; 7 received etoposide-based combination chemotherapy; 21 received single-agent methotrexate, or methotrexate with or followed by actinomycin D. Twenty-three cases (37%) received no surgical or chemotherapeutic intervention. Regardless of the therapy provided, in every case, low levels of circulating hCG persisted, and clinically this was perceived as failed therapy. Table 2 outlines a representative case history (case 2 from the Table 1). Of note, this patient had low hCG titers that persisted for at least 2½ years despite aggressive therapy including chemotherapy and hysterectomy.

Table 2. Representative case with persistent low levels of serum hCG		
Time (days)	Serum hCG (IU/L)	Notes
0	44	- incidental pregnancy test
14	40	- D&C reveals no evidence of pregnancy
21	38	- laparoscopy reveals no ectopic pregnancy
72	52	
79	38	- CT/MRI scans reveal no evidence of disease
110	40	
132	39	
177	36	
222	30	- CT/MRI scans reveal no evidence of disease
240	32	
267	35	- 4 courses of methotrexate chemotherapy
301	29	
322	35	- Actinomycin D chemotherapy
346	34	
401	28	
437	20	
467	26	
510	24	
627	60	- MRI scan indicates uterine irregularities
632	60	- HYS and BSO, pathology unremarkable
642	34	
660	13	
668	20	- 3 courses EMA-CO combination therapy
695	14	
740	21	
779	20	
803	24	
808	24	- <i>"real" hCG confirmed</i> - <i>ITA not detected indicating the diagnosis of quiescent GTD</i>
<p>This patient's antecedent gestation was a normal pregnancy (case 2, Table I). hCG levels were measured using commercial available kits at treatment center. Results from the reference laboratory are shown in italics. In this case gestational trophoblastic disease was assumed from the finding of persistent low levels of hCG in the absence of pregnancy. <i>D&C</i>, dilatation and curettage; <i>CT</i>, computerized tomographic imaging; <i>MRI</i>, magnetic resonance imaging; <i>HYS</i>, hysterectomy, <i>BSO</i>, bilateral salpingoophorectomy, <i>EMA-CO</i>, multiagent etoposide, methotrexate, actinomycin D, vincristine, and cyclophosphamide chemotherapy; <i>ITA</i>, invasive trophoblastic antigen.</p>		

In four cases (6.3%), hCG titers abruptly and steeply increased, after prolonged surveillance (after 1, 2, 3, and 4.5 years, respectively) of persistent, low-level hCG titers. In 3 of the 4 cases, the antecedent event was a pregnancy, and in one case was a complete hydatidiform mole. All 4 patients were again referred to the reference laboratory for further evaluation. At this juncture, real hCG titers substantially higher than previous results were demonstrated (Table 1, cases 7, 14, 18 and 34). In one patient tumor was identified in the right lower lobe of the lung by CT imaging. Metastatic placental site trophoblastic tumor was confirmed by pathologic inspection. This patient was given EMA-CO combination chemotherapy (etoposide, methotrexate and actinomycin C alternating with cyclophosphamide and vincristine) without complication. This suppressed the hCG to undetectable levels (<2 IU/L). In the 3 other cases with much higher rising hCG results (489, 865 and 6000 IU/L at time of re-referral), tumor was imaged in the uterus by magnetic imaging in one case. In the other 2 cases CT and magnetic resonance imaging did not identify a tumor. In all 3 cases, considering the rapidly rising hCG, invasive gestational trophoblastic neoplasm or choriocarcinoma was clinically suspected. One of patient was treated with EMA-CO, one with Actinomycin D and the third with methotrexate. In all 3 cases there were no complications and the chemotherapy, at this time, reduced the circulating hCG to undetectable levels.

In addition to intact hCG, free β -subunit and β -subunit core fragment, ITA was also measured in the most recent 38 (60%) cases. In 35 (92%), ITA was either not detectable, or accounted for $<5\%$ of hCG immunoreactivity (Figure 1). This finding indicated the absence of invasive cytotrophoblast cells. The remaining 3 cases were found to have minimal proportions of ITA accounting for 9%, 10% and 21% of total hCG immunoreactivity. In contrast, the 4 patients re-referred to the reference laboratory with rapidly rising hCG titers were found to have high proportions of ITA accounting for 81%, 86%, 91% and 100% of the detected hCG immunoreactivity. In all 4 cases, invasive gestational trophoblastic neoplasm or trophoblastic malignancy was proven or suggested. Because of these findings, the serum from another 15 women with pathologically-confirmed trophoblastic malignancy including invasive placental site trophoblastic tumor (1) and choriocarcinoma (14) were analyzed for the presence of ITA. In 10 (67%), ITA

accounted for approximately 100% of the total hCG immunoreactivity, and in all cases, exceeded 30% of the immunoreactivity (Figure 1). These results indicate that ITA has the sensitivity and specificity to differentiate a pre-invasive form with low hCG titers, or quiescent gestational trophoblastic disease, from malignant or invasive gestational trophoblastic disease.

In all cases, the diagnosis of persistent gestational trophoblastic disease was rendered, although no tumor could be identified by CT or magnetic resonance imaging. Most cases (64%) received single or multi-agent chemotherapy, surgery, or a combination. Despite therapy, measurable circulating hCG persisted. In 38 of 38 cases evaluated by the ITA assay, no or minimal immunoreactivity was identified. This indicated the absence or minimal presence of invasive cytotrophoblastic cells.

Since no discernable tumor was identified, these observations suggest that the persistent low levels of hCG were arising within slow-growing non-invasive trophoblast cells, such as fully differentiated syncytiotrophoblastic cells. It is hypothesized, without wishing to be bound by theory, that fully differentiated syncytiotrophoblastic cells are the source of the persistent hCG, based upon their limited response to chemotherapy, the absence or minimal proportions of ITA, the production of regular intact hCG, and the absence of clinically recognizable tumor. Although these cells have not been specifically identified, presumably they are late pregnancy trophoblast cells that persisted after parturition or evacuation of hydatidiform mole. Recent studies have demonstrated that in some cases the pregnancy associated with the development of gestational trophoblastic disease is not always the one most temporally related (9). In 12 cases, hysterectomy failed to completely suppress circulating hCG, which indicates that some of these cells spread outside the uterus. Although the mechanism is unknown, these cells may have been disseminated by retrograde flow, as has been described for endometriosis. Alternatively, these cells may spread by direct invasion or within lymphatic or vascular spaces, similar to other neoplastic processes involving the mullerian tract. It is further postulated that the presence of persistent quiescent hCG in women with no evidence of clinically detectable tumor constitutes a preinvasive condition that warrants observation without therapy.

In 4 cases, after 1, 2, 3, and 4½ years of dormant gestational trophoblastic disease, there was a consistent steep rise in circulating hCG levels, coincident with the development of invasive gestational trophoblastic disease. These results suggest that these cells retain the propensity to retrodifferentiate or transform into invasive cytotrophoblastic, or cytotrophoblastic-like cells. Of note, all 4 invasive cases were recorded only after re-referral to the reference laboratory. Since there is currently no required follow-up following initial consultation, the number of other cases that subsequently developed malignant disease, but were not referred back, is unknown.

These results indicated that measurement of ITA could distinguish quiescent gestational trophoblastic disease from invasive disease.

Example 2

hCG/ITA tests in Management of Gestational Trophoblastic Diseases

Forty cases were identified at the USA hCG Reference Service (Albuquerque, New Mexico) with low values of real (not false positive) hCG persisting with small variations in hCG results for period ranging from 4 months to 12 years (Table 3). In all cases no tumor was found by ultrasound, CT or MRI scans. In each case persistent gestational trophoblastic disease had been assumed, and in 25 of the 40 cases (63%) referred to the hCG Reference Service (no information available on 2 of the 40 cases) chemotherapy, combination chemotherapy (EMACO) or hysterectomy had been performed. In all cases the therapy failed to completely suppress the hCG values. In all 40 cases real hCG was detected in serum.

Table 3

Case	Time period of persistent low hCG	Limits of variable hCG values over time period	Additional therapy after persistent low hCG results identified
1	<½ year	6 – 37 IU/L	HYS, SO, EMACO, Taxol, CarP
2	>½ year	5 – 10 IU/L	AcD, Mtx, HYS, EMACO

3	?	100 – 400 IU/L	HYS, BSO, AcD
4	1½ year	<2 – 39 IU/L	AcD, EMACO, VIP, ICE
7	12 years	3 – 16	Mtx, HYS, EMACO
5	1½ year	97 – 150 IU/L	HYS, Etop
6	2 years	17 – 48	Mtx, AcD, HYS
8	1 year	6 – 54 IU/L	HYS
9	6 year	7 – 35 IU/L	Mtx, AcD
10	1 year	8 – 47 IU/L	Mtx, AcD
11	1 year	4 – 36 IU/L	Mtx, AcD
12	<½ year	19 – 32 IU/L	Mtx
13	<½ year	<5 – 12 IU/L	AcD, Mtx
14	3 year	<2 – 59 (6000 IU/L) ¹	BSO, Mtx
15	1½ year	<2 – 41 IU/L	Mtx, AcD
16	1 year	20 – 56 IU/L	Mtx, AcD
17	<½ year	7 – 48 IU/L	Mtx, AcD
18	1 year	36 – 66 IU/L	Mtx, AcD
19	<½ year	7 – 34 IU/L	Mtx, AcD
20	4½ year	2 – 7 IU/L	AcD
21	1½ year	8 – 35 IU/L	5FU
22	1 year	6 – 13 IU/L	Mtx
23	1 year	25–37 IU/L (489 IU/L) ²	Mtx
24	1 year	15 – 33 IU/L	Mtx
25	2 years	4 – 24 IU/L	Mtx
26	1 year	<2 – 23 IU/L	?
27	1 year	8 – 23 IU/L	?
28	2½ year	32 – 100 IU/L	no further cancer therapy
29	1 year	2 – 10 IU/L	no further cancer therapy
30	½ year	4 – 15 IU/L	no further cancer therapy
31	½ year	6 – 12 IU/L	no further cancer therapy
32	<½ year	49 – 64 IU/L	no further cancer therapy
33	1 year	11 – 34 IU/L	no further cancer therapy
34	½ year	14 – 21 IU/L	no further cancer therapy
35	1 year	12 – 44 IU/L	no further cancer therapy
36	<½ year	<2 – 28 (350 IU/L) ³	no further cancer therapy
37	<½ year	5–29 IU/L	no further cancer therapy
38	3 year	94–213 IU/L (213 IU/L) ⁴	no further cancer therapy
39	<0.5 year	<2 – 57 IU/L	no further cancer therapy
40	<0.5 year	<2 – 7	no further cancer therapy

¹ After 3 years of persistent low levels of hCG values started to sharply rise. Patient was referred to the USA hCG Reference Service. hCG result was 6000 IU/L. At this time

81% of the total hCG immunoreactivity was shown to be due to hyperglycosylated hCG (Table 4). Malignant disease (gestational trophoblastic disease/choriocarcinoma) was identified and chemotherapy commenced.

2 After 1 year of persistent low levels of hCG values started to sharply rise. Patient was referred to the USA hCG Reference Service. hCG result was 489 IU/L. At this time 100% of the total hCG immunoreactivity was shown to be due to hyperglycosylated hCG (Table 4). Malignant disease (gestational trophoblastic disease/choriocarcinoma) was indicated and chemotherapy commenced.

3 A repeat serum and urine sample were requested by the USA hCG Reference Service if hCG results started sharply rising. After 3 months further samples were received, levels rose to 350 IU/L. While on the first occasion, when hCG result was 22 IU/L and no hyperglycosylated hCG was detected. At time of second sample, hCG results was 194 IU/L in our assay and 57% of hCG immunoreactivity was shown to be due to hyperglycosylated hCG (Table 4). Invasive disease was indicated. Raised hCG was suppressed by EMA-CO chemotherapy.

4 During 3 years of persistent low levels of hCG, patient was referred to the USA hCG Reference Service twice. On first occasion, after 1 year of persistent low hCG results, hCG result was 44 IU/L and no ITA was detected. At time of second referral, after 3 years of persistent low hCG results, hCG results was 213 IU/L. At this time, however, lung nodule identified by CT scan, and 65% of hCG immunoreactivity was shown to be due to hyperglycosylated hCG (Table 4). Malignant disease identified and chemotherapy started.

The USA hCG Reference Service found in these cases neither detectable (<2 IU/L) nor significant proportions of ITA, as measured using the Advantage ITA test (Nichols Institute Diagnostics) (<30% of all hCG forms), in serum samples (Table 4). This observation, the lack of clear response to chemotherapy, and the absence of an identifiable mass together suggested that the low levels of hCG may have arisen from spread out, non-invasive cells remaining from a hydatidiform mole or other source of trophoblast cells. It was inferred that the pregnancy-like hCG produced by scattered differentiated trophoblast cells remaining from evacuation of hydatidiform mole or other source of trophoblast cells, were non-invasive cells, and that because they were slow growing, did not respond to chemotherapy.

Table 4

Total serum hCG	Hyperglycosylated hCG
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immunoreactivity	
IU/L	Percentage of total hCG immunoreactivity
A. Cases with persistent low levels of hCG	
38	Not detected (<2 IU/L)
50	Not detected (<2 IU/L)
14	Not detected (<2 IU/L)
16	Not detected (<2 IU/L)
22	Not detected (<2 IU/L)
22	Not detected (<2 IU/L)
13	Not detected (<2 IU/L)
34	Not detected (<2 IU/L)
56	Not detected (<2 IU/L)
39	Not detected (<2 IU/L)
213	Not detected (<2 IU/L)
57	Not detected (<2 IU/L)
148	Not detected (<2 IU/L)
224	Not detected (<2 IU/L)
496	Not detected (<2 IU/L)
12	Not detected (<2 IU/L)
21	Not detected (<2 IU/L)
20	Not detected (<2 IU/L)
100	Not detected (<2 IU/L)
7	Not detected (<2 IU/L)
150	Not detected (<2 IU/L)
20	Not detected (<2 IU/L)
21	Not detected (<2 IU/L)
37	Not detected (<2 IU/L)
47	Not detected (<2 IU/L)
48	Not detected (<2 IU/L)
130	Not detected (<2 IU/L)
29	Not detected (<2 IU/L)
24	Not detected (<2 IU/L)
7.7	Not detected (<2 IU/L)
10.6	Not detected (<2 IU/L)
88	Not detected (<2 IU/L)
42	Not detected (<2 IU/L)
24	Not detected (<2 IU/L)
16	Not detected (<2 IU/L)
51	Not detected (<2 IU/L)
16.5	Not detected (<2 IU/L)
74	Not detected (<2 IU/L)
90	Not detected (<2 IU/L)
9.8	Not detected (<2 IU/L)
8	Not detected (<2 IU/L)
44	Not detected (<2 IU/L)
60	Not detected (<2 IU/L)
12	Not detected (<2 IU/L)

35	Not detected (<2 IU/L)
11	Not detected (<2 IU/L)
5.9	Not detected (<2 IU/L)
35	Not detected (<2 IU/L)
35	Not detected (<2 IU/L)
750	3.9%
198	9.1%
212	10%
23	21%
B. Cases with proven malignant/invasive trophoblastic disease	
2450	32%
19950	32%
8130	39%
330	71%
670	~100%
2000	~100%
2500	~100%
5000	~100%
37500	~100%
38700	~100%
45000	~100%
90000	~100%
131250	~100%
C. Cases with history of persistent low hCG that developed malignant/invasive disease	
6000	81% ¹
489	100% ¹
350	57% ¹
213	65% ²
863	86% ²
190	100% ²
1255	100% ²

¹ Those with past history of gestational trophoblastic disease (Table 3 cases 13, 22, 35, 37).

² Those with no past history of gestational trophoblastic disease (Table 5 cases 4, 11, 19).

5

In one of the 26 cases (case 14 in Table 3), after 3 years of low levels of hCG, hCG values sharply rose. The patient was referred again to the USA hCG Reference

Service. The hCG result was 6000 IU/L. At this time 81% of the total hCG immunoreactivity was shown to be due to ITA (Table 4), consistent with invasive/malignant disease. A CT scan detected a tumor, gestational trophoblastic disease/choriocarcinoma was diagnosed and EMA-CO chemotherapy was started. As described in Table 3, three other cases (23, 36 and 38) had rising hCG results and invasive disease, accompanied by ITA to total hCG ratios of 100%, 57%, and 65%, respectively.

The USA hCG Reference service also consulted on 40 other cases with persistent low hCG values but with history of pregnancy and no history of trophoblastic diseases whatsoever (Table 5). In these cases, persistent low hCG levels were detected from 2 month to 6 years. In all 40 cases hCG was identified in both serum and urine samples. The hCG resembled normal second or third trimester pregnancy hCG, with very little or no ITA (<2 IU/L or <30% of total hCG). In all 40 cases no tumor mass was identified. Little or no response was observed to chemotherapy.

Table 5

Case	Time period of persistent low hCG	Limits of variable hCG values over time period	Therapy
1	3 year	13 – 60 IU/L	Mtx, AcD, HYS, BSO, EMACO
2	3 year	18 – 150 IU/L	HYS, BSO, AcD, EMACO
3	3½ year	11 – 19 IU/L	Mtx, AcD, HYS, BSO, AcD
4	4 year	37 – 198 IU/L (863 IU/L) ¹	MtxLv, HYS, BSO, Mtx
5	6 year	44 – 198 IU/L	HYS, Mtx, EMACO
6	2 year	<2 – 90 IU/L	Mtx, AcD, EMACO
7	2 years	5 – 130 IU/L	Mtx, AcD, HYS
8	3 year	<1 – 100 IU/L	Mtx, HYS
9	4 years	6 – 11	HYS
10	1 year	60 – 130 IU/L	HYS
11	2 year	17 – 148 IU/L (355 IU/L) ²	Mtx, AcD
12	1 year	54 – 500 IU/L	Mtx, AcD

13	<½ year	7 – 35 IU/L	Mtx, Mtx
14	<½ year	12 – 42 IU/L	Mtx, Mtx
15	<½ year	165 – 224 IU/L	Mtx
16	<½ year	6 -14	Mtx
17	<½ year	174 – 224 IU/L	Mtx
18	<½ year	34 – 56 IU/L	Mtx
19	1 year	100–174 IU/L(1255 IU/L) ³	Mtx
20	<½ year	10 – 12 IU/L	Mtx
21	2 year	50 – 200 IU/L	Mtx
22	1 year	2 – 485 IU/L	Non-specified chemotherapy
23	<½ year	10 – 148 IU/L	?
24	2 year	7 – 22 IU/L	?
25	<½ year	157 – 206 IU/L	no cancer therapy
26	<½ year	5 – 8 IU/L	no cancer therapy
27	1 year	8 – 21 IU/L	no cancer therapy
28	3 year	4 – 7 IU/L	no cancer therapy
29	<½ year	58 – 146 IU/L	no cancer therapy
30	<1 year	2 -10 IU/L	no cancer therapy
31	<1 year	5 – 10 IU/L	no cancer therapy
32	2 year	10 – 40 IU/L	no cancer therapy
33	<½ year	223 – 496 IU/L	no cancer therapy
34	<½ year	6 – 9 IU/L	no cancer therapy
35	<½ year	6 – 7 IU/L	no cancer therapy
36	<½ year	14 -22 IU/L	no cancer therapy
37	5½ year	23 – 52 IU/L	no cancer therapy
38	<½ year	8 – 11 IU/L	no cancer therapy
39	<½ year	49 – 69 IU/L	no cancer therapy
40	1 year	<2 – 20 IU/L	no cancer therapy

1 After 4 years of persistent low levels of hCG (hyperglycosylated hCG not detected), values sharply rose. USA hCG Reference Service was consulted. hCG was 863 IU/L, 86% due to hyperglycosylated hCG (Table 4). Malignancy (gestational trophoblastic disease/choriocarcinoma) was demonstrated.

2 After 2 years hCG results increased to 190 IU/ML. At that time ~100% was due to hyperglycosylated hCG (Table 4). Malignancy (PSTT) was demonstrated.

3 After 1 year of persistent low levels of hCG (hyperglycosylated hCG not detected), values sharply rose. USA hCG Reference Service was consulted. hCG was 1255 IU/L, ~100% due to hyperglycosylated hCG (Table 4). Malignancy (gestational trophoblastic disease/choriocarcinoma) was demonstrated.

Most of the unexplained elevated hCG cases, like the false positive hCG cases, started with an incidental hCG test, ultrasound to exclude intra-uterine pregnancy, and dilation curettage and laparoscopy to exclude extra-uterine pregnancy. Patients were monitored with either no firm diagnosis or gestational trophoblastic disease was assumed.

- 5 Twenty-two of the 40 (55%) cases received single agent chemotherapy, combination chemotherapy, or had a hysterectomy or other surgery (Table 5). The hCG values were not effectively abated by the therapy. A representative case is outlined in Table 6 (Table 4, case 1).

10

Table 6

Time (days)	hCG (IU/L)	Notes
0	44	- incidental preg test
14	40	- D&C reveals no evidence of pregnancy
21		- laparoscopy reveals no ectopic pregnancy
72	52	
222	30	- CT/MRI scans reveal no disease
240	32	
267	35	- 4 courses Mtx chemotherapy
301	29	
322	35	- AcD chemotherapy
420	34	
627	60	- MRI scan indicates uterine irregularities
632	60	- HYS and BSO, pathology unremarkable
642	34	
668	20	- 3 courses EMACO combination therapy
803	24	
808	24	- real hCG confirmed no hyperglycosylated hCG detected, unexplained elevated hCG indicated.

- 15 In three of the 40 cases (cases 4, 11, and 19 in Figure 5Table 4), after 1, 2, and 4 years of varying low levels of hCG results (ITA was not detected), values started to sharply rise. The USA hCG Reference Service was again consulted. In one case the hCG was 190 IU/L, in the second 863 IU/L, and the third 1255 IU/L. ITA measurements

were consistent with invasive/malignant disease (86%, ~100%, and ~100% of total hCG immunoreactivity). Tumor was identified by MRI/CT imaging. In the first case placental site trophoblastic tumor (PSTT) was identified by pathology and in the second and third cases gestational trophoblastic disease/choriocarcinoma was diagnosed. All three were treated with EMA-CO chemotherapy.

In summary, the USA hCG References Service observed 80 cases of persistent low levels of hCG, whether with history of hydatidiform mole or gestational trophoblastic disease (quiescent gestational trophoblastic disease) or with history of only pregnancy (unexplained elevated hCG). In 7 of the 80 cases (those referred for a second time to the USA hCG Reference Service) after periods of 2 months to 6 years of persistent low hCG levels, hCG results rapidly rose, indicating active disease. In one case PSTT was detected and in six cases gestational trophoblastic disease/choriocarcinoma was diagnosed and treated. This observation indicated that persistent low levels of hCG may be a pre-malignant condition.

Total hCG (DPC Immulite) and ITA (Nichols Institute Diagnostics: Advantage ITA test) were measured in 53 of the 80 cases with persistent low hCG levels (Tables 7 and 5). The proportion of total hCG due to ITA was determined. As shown in Table 4, ITA was not detected (<2 IU/L) in 49 of the 53 cases with persistent low levels of hCG, and accounted for no more than 21% (<30%) of the total hCG in the remaining four cases. Sera from 13 additional patients with proven choriocarcinoma were also examined. In all cases, only high proportions of ITA (>30% of total hCG) were detected (Table 4).

As described in Tables 7 and 3, in 7 of the 80 cases with persistent low levels of hCG, malignant disease developed. ITA results were 57% to ~100% of total hCG (Table 4). Putting these cases together, ITA accounted for more than 30% of total hCG immunoreactivity in 20 of 20 malignant cases, and in none of the 53 individuals with persistent elevated hCG (non-invasive disease). The data indicated that the percent ITA is an accurate indicator of invasive/malignant trophoblastic disease, with use in differentiating persistent low levels of hCG from malignant disease.

Table 7

Case	Treatment given because of persistent false positive hCG result	False hCG result recorded by physician at time of hCG Reference Service testing
1	D&C, Lap, Mtx, AcD, HYS, EMACO, THO	220 IU/L hCG
2	D&C, Lap, Mtx, HYS, EMACO, Coma	68 IU/L hCG
3	D&C, Lap, Mtx, Mtx, AcD, HYS	142 IU/L hCG
4	D&C, Lap, Mtx, HYS	17 IU/L hCG
5	D&C, Lap, HYS, BSO	150 IU/L hCG
6	D&C, Lap, Mtx, BSO	145 IU/L hCG
7	D&C, Lap, Mtx, HYS, BSO	81 IU/L hCG
8	D&C, Lap, Mtx, AcD, EMACO	558 IU/L hCG
9	D&C, Lap, Mtx, EMACO	80 IU/L hCG
10	D&C, Lap, Mtx, AcD	22 IU/L hCG
11	D&C, Lap, Mtx, AcD	21 IU/L hCG
12	D&C, Lap, Mtx, AcD	110 IU/L hCG
13	D&C, Lap, Mtx, AcD	114 IU/L hCG
14	D&C, Mtx, D&C, Lap, Mtx	24 IU/L hCG
15	D&C, Lap, Mtx, Mtx	97 IU/L hCG
16	D&C, D&C, Lap, Mtx	122 IU/L hCG
17	D&C, Mtx, ActD	14 IU/L hCG
18	D&C, Lap, Mtx	60 IU/L hCG
19	D&C, Lap, Mtx	32 IU/L hCG
20	D&C, Lap, Mtx	402 IU/L hCG
21	D&C, Lap, Mtx	37 IU/L hCG
22	D&C, Lap, Mtx	607 IU/L hCG
23	D&C, Lap, Mtx	300 IU/L hCG
24	D&C, Lap, Mtx	170 IU/L hCG
25	D&C, Lap, Mtx	21 IU/L hCG
26	D&C, Mtx	20 IU/L hCG
27	D&C, Mtx	202 IU/L hCG
28	D&C, Mtx	18 IU/L hCG
29	D&C, Mtx	23 IU/L hCG
30	D&C, Mtx	81 IU/L hCG
31	D&C, Mtx	32 IU/L hCG
32	D&C, Mtx	170 IU/L hCG
33	D&C, Mtx	12 IU/L hCG
34	D&C, Mtx	124 IU/L hCG
35	D&C, Mtx	70 IU/L hCG

36	D&C, Mtx	21 IU/L hCG
37	D&C, Mtx	17 IU/L hCG
38	D&C, Lap	14 IU/L hCG
39	D&C, Lap	23 IU/L hCG
40	D&C, Lap	24 IU/L hCG
41	D&C, Lap	53 IU/L hCG
42	D&C	32 IU/L hCG
43	D&C	93 IU/L hCG
44	D&C	143 IU/L hCG
45	-	25 IU/L hCG
46	-	18 IU/L hCG
47	-	7 IU/L hCG
48	-	224 IU/L hCG
49	-	20 IU/L hCG
50	-	18 IU/L hCG
51	-	25 IU/L hCG
52	Male patient, testicular cancer considered	38 IU/L hCG

Example 3

Presence of ITA in Germ Cell Cancers

5 Three patients suspected of having seminoma or testicular choriocarcinoma, both of which are testicular germ cell cancers, were referred to the USA hCG Reference Service. Samples of urine and serum were obtained from each patient and the level of total hCG was analyzed using DPC Immulite test kit. The samples were then tested for immunoreactivity to ITA using the Nichols Institute Diagnostics: Advantage ITA test. In
10 all three cases, all of the hCG immunoreactivity was due to ITA, showing that these testicular germ cell cancers, like placenta choriocarcinoma, produce ITA.

 In addition, five patients diagnosed as having ovarian germ cell malignancies (dysgerminoma) were referred to the hCG Reference Service. Samples from these five patients were also analyzed for total hCG titers (DPC Immulite), followed by testing for
15 ITA immunoreactivity (Nichols Institute Diagnostics: Advantage ITA test). All of the hCG immunoreactivity in these patients was due to ITA. Thus, ITA serves as a useful marker to detect both testicular and ovarian germ cell cancers.

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The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.